REMARKS

Applicant has amended the specification to include the SEQ ID NO designation assigned to a particular sequence when the corresponding sequence is referred to in the specification. The incorporated SEQ ID NOs correspond to those designated by the substitute sequence listing submitted on December 6, 2001. The specification has also been amended to properly refer to the figures by their appropriate designations. The amendments to the specification do not add new matter.

Respectfully submitted,

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Title: Isolated Yeast Promoter Sequence and a Method of Regulated Heterologous Expression

VERSION WITH MARKINGS TO SHOW CHANGES MADE ACCOMPANYING PRELIMINARY AMENDMENT

In the Specification

The replacement specification paragraphs incorporate the following amendments. Underlines indicate insertions and ~~strikeouts~~ indicate deletions.

The section entitled Brief Description of the Drawings, beginning at page 5, line 22, has been amended as follows:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration depicting an inverse PCR method for promoter clone isolation.

FIG. 2 is a photograph of a reverse gel image of PCR clones of *S. castellii* glucoamylase promoter.

FIG. 3 is a schematic illustrating the construction of a plasmid vector pGA2066.

FIG. 4 is the sequence of *S. castellii* glucoamylase promoter; SEQ ID NO:8.

FIG. 5 is the sequence comparison of two *S. castellii* glucoamylase promoter sequences; SEQ ID NO:9 (top sequence) and SEQ ID NO:10 (bottom sequence).

FIG. 6 is a schematic illustrating the construction of a plasmid vector pGA2100.

FIG. 7 is a schematic illustrating the construction of a plasmid vector pGA2101.

The paragraph beginning at line 23 on page 6 has been amended as follows:

However, other reports showed that a glucoamylase structure gene of a similar *S. castellii* strain (ATCC 26076), which is under the control of a native GAM promoter, was unable to express in the transformed *Saccharomyces cerevisiae* host while this glucoamylase gene was able to express under the control of *S. cerevisiae* promoters such as galactokinase (GAL1) promoter. This is probably due to the difference in the 5' flanking region of the glucoamylase gene of these two *S. castellii* strains. When compared to the GAM gene 5' end region of a *S. castellii* strain (ATCC 26076), it was found that there are sequence differences at positions 160-162 bps, 168-169 bps, and 288 bp using GAM76 SEQ ID NO:10, as basis as shown in Figure 5 ~~4(C)~~, where GAM76 stands for the GAM 5' end flanking region of *S. castellii* (ATCC 26076) and GAM77 for the GAM 5' end flanking region of *S. castellii* (ATCC 26077). In GAM77 SEQ ID NO:9, there is a sequence for CCATTATGGAT as compared to the difference of CATATGGTA in GAM76, which might cause inactivation of GAM76 in *S. cerevisiae*. However, there was no comparison made beyond 325 bps upstream of the initiation codon between two glucoamylase genes since the 5' end sequence beyond 325 bps upstream is not available for *S. castellii* (ATCC 26076).

The paragraph beginning at line 6 on page 8 has been amended as follows:

To isolate the glucoamylase promoter, *S. castellii* cells were grown overnight in a culture medium containing yeast extract 1%, peptone 2%, and glucose 2%. Cells were then harvested and genomic DNA was isolated and purified from the culture using the spheroplasting method. Inverse PCR method was used to clone out the promoter region, as shown in Figure 1 where P1 is PCR reverse primer 1; P2 PCR forwarding

primer 2; RE restriction enzyme site which can be cleaved both upstream of the glucoamylase (GAM) promoter and inside of the GAM gene; SCst *S. castellii.* PCR primers for the inverse PCR were designed based on the open reading frame of the GAM gene sequence of *S. castellii.* A 5' end over-hung sequence (italics) was designed to adapt restriction enzyme sites (underlined) such as Xba I and Sph I. The inverse PCR primers are listed as following:

Reverse primer GL1-C61:SEQ ID NO:1

5'-*GC TCT AGA* CAT ATG AGT AGT TTC CGT AGT AAT TGA-3'

Reverse primer GL2-C62: SEQ ID NO:2

5'- *GC TCT AGA* ATT ACT ATA CTT TTA ATC AGC TTC AGA-3'

Forwarding primer GL1-N64: SEQ ID NO:3

5'-*GAT GCA TGC* TAT CTT TAA TGA CTC TGC TGT CGA TGC -3'

Forwarding Primer GL3-N66: SEQ ID NO:4

5'-*GAT GCA TGC* TAG TTG TTA AAC CAC TGG TGG AAG GTG-3'

The paragraph beginning at line 7 on page 10 has been amended as follows:

PCR product No. 2 contains sufficient length (about 2.3 kb) of the GAM gene upstream sequence and the initial codon ATG of the GAM gene. The GAM promoter

clone No. 2 was cloned into a unique cloning site containing 3' terminal thymidine (T) to both ends of a cloning plasmid vector pGEM-T (Promega, Madison, WI) to form pGA2066, as shown in Figure 3, where Amp is the ampicillin resistance gene; ColE1 is the origin for plasmid replication during gene manipulation in *E. coli* strains; f1 ori is the phage origin. Individual colonies were picked to confirm DNA insertion. Two of the randomly picked individual clones, pGA2066-21 and pGA2066-29, were sequenced to compare the identity of these two clones. The results showed that these two clones have the same sequences. In addition, other clones from PCR products No. 8 and 11 were also sequenced and they had the identical sequences as that of No. 2, and the sequence downstream of the initiation codon ATG is identical to the GAM1 gene. Therefore, the upstream of clone No. 2 has the GAM promoter region. Clone No. 2 in pGA2066-21 was chosen and completely sequenced from both ends, and has a length of 2182 bp, corresponding to SEQ ID NO:8, as shown in Figure 4 ~~(A) and Figure 4(B)~~. The putative TATA box and CAT box are bold and underlined. There are seven CAT boxes and ten TATA boxes within 600 base pairs upstream of the initial codon. In addition, the open reading frame analysis indicates that the GAM promoter sequence locates between 485 bp to 2148 bp, between which there are 1662 bps for the GAM promoter and there is no long open reading frame.

The paragraph beginning at line 28 on page 10 has been amended as follows:

To test the activity of the glucoamylase (GAM) promoter, a bacterial glucuronidase gene was fused to the 1.5 kb and 1.0 kb glucoamylase promoters. An episomal yeast plasmid vector pGA2028D was used, as shown in Figure 6 ~~5~~. The GAM promoter was cloned out from pGA2066-21, forming the 1.5 kb and 1.0 kb GAM promoters (GAM15 and GAM10). A 5' end over-hung sequence (italics) was designed

to adapt restriction enzyme sites (underlined) such as Spe I at the 5' end and Hind III at the 3' end, using the following primers, respectively.

Forwarding primer GM15-N for 1.5 kb GAM promoter: SEQ ID NO:5

5'-*TCT AGA ACT AGT* GAT TTC TGA TTG ATT TGA GTT-3'

Forwarding primer GM10-N for 1.0 kb GAM promoter: SEQ ID NO:6

5'-*TCT AGA ACT AGT* TCT ATC AAA CTA CTC CAA ATA-3'

Reverse primer GM-C for both GAM promoters: SEQ ID NO:7

5'-*GGT ACC AAG CTT* CTT GCT TGT TAT AAT ACA GTC-3'

The paragraph beginning at line 15 on page 11 has been amended as follows:

Figure 6 ~~5~~ shows the constructed vector pGA2100 containing the 1.5 kb GAM promoter, and Figure 7 ~~6~~ shows the vector pGA2101 containing the 1.0 kb GAM promoter, where 2 micron is a DNA replicon for plasmid replication in *Saccharomyces* strains; ColE1 is the origin for plasmid replication during gene manipulation in *E. coli* strains; f1 ori is the phage origin; *gus* is the bacterial glucuronidase gene; Tcyc1 is the transcription terminator; and Zeocin is the Zeocin resistance gene (Invitrogen, Inc, Carlsbad, CA). pGAM15 and pGAM10 stand for the GAM promoter with lengths of 1.5 kb and 1.0 kb, respectively.

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